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## MUSCULOSKELETAL PATHOLOGY

# Association between Periodontal Disease and Inflammatory Arthritis Reveals Modulatory Functions by Melanocortin Receptor Type 3

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Because there is clinical evidence for an association between periodontal disease and rheumatoid arthritis, it is important to develop suitable experimental models to explore pathogenic mechanisms and therapeutic opportunities. The K/BxN serum model of inflammatory arthritis was applied using distinct protocols, and modulation of joint disruption afforded by dexamethasone and calcitonin was established in comparison to the melanocortin (MC) receptor agonist DTrp<sup>8</sup>— $\gamma$ -melanocyte stimulating hormone (MSH; DTrp). Wild-type and MC receptor type 3 (MC<sub>3</sub>)-null mice of different ages were also used. There was significant association between severity of joint disease, induced with distinct protocols and volumes of the arthritogenic K/BxN serum, and periodontal bone damage. Therapeutic treatment with 10  $\mu$ g dexamethasone, 30 ng elcatonin, and 20  $\mu$ g DTrp per mouse revealed unique and distinctive pharmacological properties, with only DTrp protecting both joint and periodontal tissue. Further analyses in nonarthritic animals revealed higher susceptibility to periodontal bone loss in *Mc3r*<sup>-/-</sup> compared with wild-type mice, with significant exacerbation at 14 weeks of age. These data reveal novel protective properties of endogenous MC<sub>3</sub> on periodontal status in health and disease and indicate that MC<sub>3</sub> activation could lead to the development of a new genus of anti-arthritic bone-sparing therapeutics. (*Am J Pathol* 2014, 184: 2333–2341; <http://dx.doi.org/10.1016/j.ajpath.2014.04.009>)

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with progressive disability, early death, increased risk of cardiovascular events, and other extra-articular manifestations that have a major impact in the quality of life of those with the disease.<sup>1,2</sup> The current clinical approach is to start early, after diagnosis, with aggressive therapy, followed by treatment adjustments according to changes in disease activity. However, despite the important progress in RA therapies during the past decade, several needs are still unmet. The introduction of biological agents in the early 1990s revolutionized the treatment of RA and other chronic diseases, such as inflammatory bowel disease. However, although highly effective and generally faster acting than disease-modifying anti-rheumatic drugs, many patients are not responsive, and they may also experience an

increased risk of opportunistic infections. The treatments are costly.<sup>3</sup> Thus, there is justification for exploiting novel therapies. In addition, it is also desirable to produce new therapeutics with efficacy on pain and inflammation in the joint, but also able to temper systemic complications of RA affecting the heart, lungs, muscles, and bone.<sup>1</sup>

Targeting the melanocortin (MC) system<sup>4</sup> to treat RA may represent an alternative opportunity to drug discovery.<sup>5</sup> Indeed, one of the melanocortin agonists, adrenocorticotropin hormone (ACTH), was shown to be effective in

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human RA >60 years ago<sup>6</sup>; researchers are showing a renewed interest.<sup>7</sup> This is prompted by the fact that ACTH may afford biological actions beyond the endogenous production of cortisol,<sup>8,9</sup> provoking activation of peripheral MC receptors, including the melanocortin receptor 3 (MC<sub>3</sub>). This peripheral mechanism of action of ACTH, hence independent from adrenal release of glucocorticoids, might also underlie efficacy in conditions such as proteinuric nephropathies<sup>10</sup> and multiple sclerosis.<sup>11</sup>

Surmounting evidence indicates an important counter-regulatory role for the melanocortin pathway during inflammation, including in the osteo-articular system, where melanocortin receptors are expressed by osteoblasts, osteoclasts, chondrocytes, fibroblasts, and immune cells. Pharmacological targeting with MC peptides leads to a variety of protective actions, including increased matrix deposition, reduced fibroblast activation, and osteoblast and chondrocyte proliferation.<sup>12–17</sup> *In vivo*, the synthetic peptide DTrp<sup>8</sup>– $\gamma$ -melanocyte stimulating hormone (MSH; DTrp) reduces clinical signs of disease in models of inflammatory arthritis<sup>18</sup> and urate crystal peritonitis<sup>19</sup> by a mechanism involving MC<sub>3</sub>. In addition, the pan-MC agonist peptide AP214 also displays anti-arthritis properties.<sup>20</sup> Recent work by Gomez-SanMiguel et al<sup>21</sup> reported that the MC agonist  $\alpha$ MSH can reduce joint inflammation, together with an improvement of extra-articular signs associated with systemic arthritis, by increasing body weight and reducing levels of muscle-wasting markers.

An important clinical manifestation associated with arthritis is periodontal disease. There is epidemiological evidence associating inflammation of the gum with incidence of RA<sup>22</sup> and, conversely, there is a higher incidence of periodontitis in RA patients.<sup>23</sup> Intriguingly, recent reports demonstrated the presence of alveolar bone loss, an important feature of periodontitis, in rodents during the time course of experimental models of arthritis, namely collagen- and adjuvant-induced arthritis.<sup>24–26</sup> Herein, we investigated the presence of alveolar bone loss in a different model of experimental arthritis: one induced by the arthritogenic K/BxN serum, which is much faster in its kinetics, and is characterized by leukocyte infiltration, synovocyte proliferation, and cartilage and bone erosion, thus resembling many features of human RA in its active flares.<sup>27,28</sup> In addition, we established the involvement of the melanocortin system in the development of alveolar bone loss by using a combination of genetically engineered mice and pharmacological approaches.

## Materials and Methods

### Animals

The 7- to 8-week-old male mice were maintained on a standard chow pellet diet and had free access to water with a 12-hour light-dark cycle. C57BL/6J wild-type (WT) mice were purchased from Charles River (Kent, UK). *Mc3r*<sup>-/-</sup> mice were a generous gift of Dr. Howard Chen (Merck Laboratories, Rahway, NJ). All animal studies were approved and

performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and The London School of Medicine (London, UK), and Home Office Regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

### Production of K/BxN Serum

K/BxN mice were produced by crossing the C57BL/6 mice (carrying the KRN homozygously) and the NOD/Lt mice (carrying the A<sup>g</sup>7 allele homozygously).<sup>27</sup> The offspring develop spontaneous arthritis, evident at 6 weeks, with 100% incidence. At 9 weeks of age (when the titers of anti-glucose-6-phosphate isomerase antibodies are maximal, implying potent arthritogenic properties of the serum), mice were exsanguinated by cardiac puncture under anesthesia (isoflurane). Blood was allowed to clot overnight at 4°C. Serum was recovered with a Pasteur pipette and centrifuged 10 minutes at 500  $\times$  g at 4°C. The serum from different mice obtained on a given day was pooled, divided into aliquots, and stored at –80°C until use.

### K/BxN Serum Transfer Arthritis Model

Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice. Three different protocols were studied: i) protocol 50 + 50, where mice received two injections of 50  $\mu$ L of serum on days 0 and 2; ii) protocol 100 + 100, where mice received two injections of 100  $\mu$ L of serum on days 0 and 2; and iii) protocol 200, consisting of one single injection of 200  $\mu$ L of serum on day 0. The protocol 100 + 100 was then selected for subsequent experiments. The development of the disease was monitored daily by assessing the paw volume using a plethysmometer (Ugo Basile, Comerio, Italy), body weight, clinical score (score per paw: 0, no signs of inflammation; 1, subtle inflammation, localized; 2, easily identified inflammation, but localized; 3, evident inflammation, not localized; maximum score = 12 per mouse), and disease incidence [mice showing overt signs of joint inflammation (ie, a clinical score of  $\geq 1$ )].<sup>29</sup> Severe arthritis (number of paws per mouse that reached a maximum score of 3) was also recorded.

### Pharmacological Treatments

Mice ( $n = 5$ ) were treated i.p. once daily, starting from day 2 (1 hour after the second K/BxN injection), with 10  $\mu$ g per mouse dexamethasone (Dex; Sigma, Poole, UK), 20  $\mu$ g/mouse DTrp (American Peptide, Sunnyvale, CA), 30 ng per mouse elcatonin (ECT; Bachem, Bubendorf, Switzerland), or vehicle (PBS). Doses were selected from previous studies in this or similar rodent models.<sup>18,30,31</sup>

### Measurement of Alveolar Bone Loss

Alveolar bone loss was evaluated as previously described.<sup>32</sup> Mice were euthanized and maxillae were hemisected, exposed overnight in 3% hydrogen peroxide, mechanically defleshed, and stained with 0.3% methylene blue. Images of

the palatal faces of the molars were obtained using a stereomicroscope and a digital camera (Kodak EasyShare C743; Rochester, NY). Quantitative analyses included the measurement of the area between the cement enamel junction and the alveolar bone crest in the first molar, using ImageJ software version 1.46e (NIH, Bethesda, MD).

### Activity Assay

Myeloperoxidase (MPO) activity was measured as an index of granulocyte infiltration. Briefly, maxillae tissue samples were homogenized in 0.5% hexadecyltrimethylammonium bromide dissolved in phosphate buffer solution (pH 6) using a Precellys24 homogenizer in Precellys lysing CK14 tubes (Bertin Technologies; distributed by VWR International, Dublin, Ireland). The homogenized tissues were centrifuged at  $13,000 \times g$  for 5 minutes (at 4°C), and the supernatants were placed on 96-well plates. Buffer, supplemented with 1% hydrogen peroxide/O-dianisidine dihydrochloride, was added to each well. Optical density readings were taken for 3 minutes at 30-second intervals at 450 nm using a microplate reader NOVOstar (BMG Labtech, Aylesbury, UK). Activity was normalized to the sample protein concentration determined with a bicinchoninic acid kit (Pierce, Cramlington, UK), and MPO activity is expressed as U/mL of homogenated samples.

### Histological Analysis

Maxillae tissues were fixed in 10% buffered formalin (pH 7.4) for 24 hours at room temperature. The specimens were demineralized in 14% EDTA for 2 weeks, dehydrated in graded ethanol, and embedded in paraffin. Serial sections (5 µm thick) were stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, St. Louis, MO). Histological osteoclast counting was performed in the coronal two thirds of the distal alveolar bone adjacent to the first molar in five consecutive microscopic fields ( $\times 40$  magnification per section). Samples were analyzed using an Axioskop 40 microscope (Carl Zeiss, Gottingen, Germany), attached to a digital camera (PowerShot A620; Canon, Tokyo, Japan). For each animal ( $n = 4$ ), three maxillae sections were analyzed. All of the slides were counted in a blinded manner by a single examiner (M.F.M.M.). For neutrophil staining, tissue specimens were blocked by incubation in 1.5% H<sub>2</sub>O<sub>2</sub> in methanol solution for 30 minutes. Primary antibody against neutrophil elastase (Santa Cruz Biotechnology, Heidelberg, Germany) was used with a Vectastain avidin-biotin complex kit anti-rabbit (Vector Laboratories, Burlingame, CA). The sections were counterstained using hematoxylin. Slides were developed using a peroxidase substrate diaminobenzidine kit (Vector Laboratories).

### Statistical Analysis

Data were analyzed by Student's *t*-test, one- or two-way analysis of variance, followed by Bonferroni or multiple-comparison

test, or two-way analysis of variance, followed by Newman-Keuls multiple-comparison test or Pearson correlation test, as appropriate. In all cases, data are presented as means  $\pm$  SEM of  $n$  independent observations and were considered statistically significant when  $P < 0.05$ .

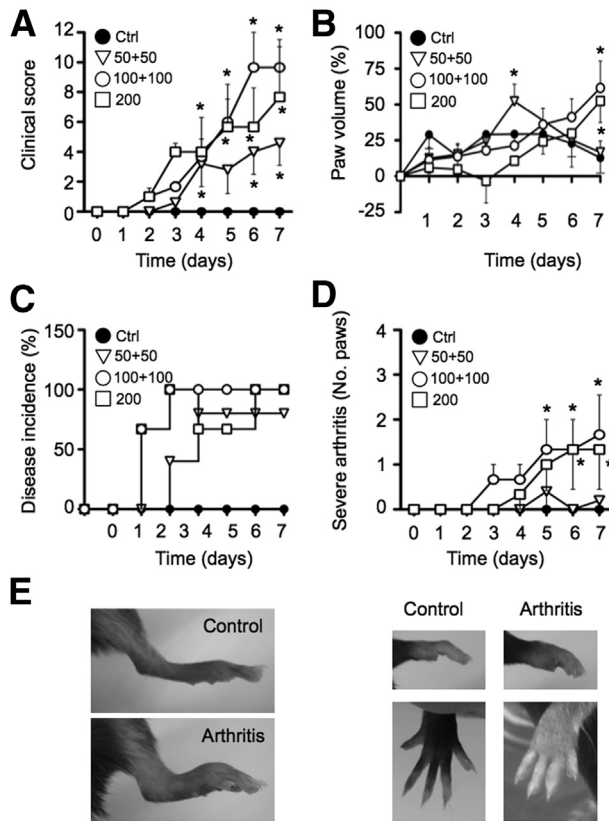
## Results

### Arthritis Severity in the K/BxN Serum Transfer Model Correlates with Alveolar Bone Loss

Recent reports have indicated that experimental models of collagen- and antigen-induced arthritis are associated with development of alveolar bone loss. We evaluated whether development of aggressive joint inflammation using the K/BxN serum transfer model of arthritis was also associated with this extra-articular manifestation. We, therefore, used three dosing strategies to manipulate the severity of arthritis: mice received two injections of either 50 or 100 µL of serum on days 0 and 2 (protocol 50 + 50 or protocol 100 + 100, respectively) or a single injection of 200 µL of serum on day 0 (protocol 200). All three regimens induced overt signs of arthritis. As expected, the arthritic response produced with protocol 50 + 50 was milder, evidenced by the low clinical score (Figure 1A), transient increase in paw volume (Figure 1B), and reduced severity (Figure 1C) compared with the other two protocols. Comparatively, although mice received the same total amount of arthritogenic serum using protocols 100 + 100 and 200, administration in two separate injections (days 0 and 2) resulted in higher clinical scores, more gradual and consistent increase in paw volume, and, more important, 100% disease incidence (Figure 1D). We next analyzed alveolar bone loss in the maxillae of these mice subjected to serum transfer-induced arthritis. Interestingly, bone loss in the maxillae was highly correlated with the severity of localized inflammation in the joints (Figure 2A), suggesting that extra-articular manifestations of relevance for RA also occur in this model. An overall comparison between nonarthritic and arthritic mice indicated a significant (21%) increase in alveolar bone loss (Figure 2B). This could also be seen macroscopically by an increased corono-apical area between the cement-enamel junction and the alveolar bone crest on the palatal side of the first molar (Figure 2C).

### Melanocortin Treatment Reduces Arthritis and Prevents Alveolar Bone Loss

The melanocortin system is widely associated with joint disease and inflammation both mechanistically and therapeutically.<sup>5,8,18,19</sup> We studied if pharmacological intervention with melanocortin-based compounds could have an impact, not only in joint inflammation or other arthritis-related manifestations, such as cachexia, as recently described,<sup>21</sup> but also in alveolar bone loss. We, therefore, assessed whether the peptide DTrp could prevent the



**Figure 1** Comparison of three protocols for the K/BxN serum transfer arthritis model. Arthritis was induced by the i.p. injection of serum from K/BxN arthritic mice using three different protocols: 50 + 50 (two injections of 50  $\mu$ L on days 0 and 2); 100 + 100 (two injections of 100  $\mu$ L on days 0 and 2); and 200 (one single injection on day 0). Clinical score (A), paw volume (B), and disease incidence (C) were recorded for 7 days. D: The number of paws per mouse that reached the maximum score (3). E: Representative images of ankle, wrist, and digit swelling. Data are the means  $\pm$  SEM of four to six mice per group. \* $P$  < 0.05, two-way analysis of variance, followed by Bonferroni multiple-comparison test. Ctrl, control.

development of alveolar bone loss we observed using this passive transfer model of arthritis. Dex, potent as an anti-inflammatory drug but with detrimental bone effects, and ECT, a bone-protective molecule but with mild anti-inflammatory effects, were used for comparison. As expected, Dex afforded a potent anti-inflammatory effect, reducing clinical score ( $-40\%$  at day 8) (Figure 3A), paw volume ( $-59\%$ ) (Figure 3B), and incidence of severe arthritis ( $-93\%$ ) (Figure 3C). The effect of ECT was less pronounced, with a significant statistical difference only in reducing paw volume. DTrp presented a moderate and significant attenuation in all parameters measured (clinical score,  $-23\%$ ; paw volume,  $-44\%$ ; severity,  $-57\%$ ).

Determination of MPO activity in maxillae tissues (analyzed at day 8) showed a significant reduction in the groups treated with either Dex or DTrp, but not with ECT (Figure 3E). Although the degree of anti-inflammatory activity attained by treatment of animals with Dex and DTrp in the periodontal tissue appears to be similar, assessment of alveolar bone loss revealed interesting differences: the

anti-arthritic effect of DTrp is associated with bone protection, as evident from the positive correlation ( $r = 0.87$ ) between bone loss and clinical score (Figure 3F). In contrast, the anti-arthritic effect of Dex was inversely correlated with alveolar bone loss ( $r = -0.87$ ), in accordance with the well-known effects of Dex on bone metabolism. These findings suggest that melanocortin therapy, in addition to potent modulation of inflammation and arthritis, could also have the advantage over corticoid therapy in preserving bone integrity.

### MC<sub>3</sub>-Deficient Mice Display Increased Alveolar Bone Loss

Our next approach consisted of the study of the role of MC<sub>3</sub> using genetically modified mice lacking this receptor, because it has been reported that MC<sub>3</sub> is a pivotal target for the anti-inflammatory and anti-arthritic actions of melanocortin drugs, including ACTH.<sup>8,9</sup> Arthritis was induced using the 100 + 100 protocol, as in previous experiments, although in this case, 12-week-old mice were used (because of stock availability). As shown in Figure 4, arthritis developed similarly to previous experiments with younger mice. The clinical score and disease severity were identical in WT and *Mc3r*<sup>-/-</sup> mice, although some differences were found in the paw volume (Figure 4). As expected, when alveolar bone loss was assessed in maxillae (day 8), WT arthritic mice presented an increase in bone loss, corroborating our previous findings. However, the basal values of alveolar bone loss obtained in *Mc3r*<sup>-/-</sup> mice were elevated compared with WT mice, and no further alveolar bone loss was obtained after arthritis induction (Figure 4D). The fact that the mice used in this experiment were older made us hypothesize that there might be an association between MC<sub>3</sub> deficiency and physiological bone metabolism.

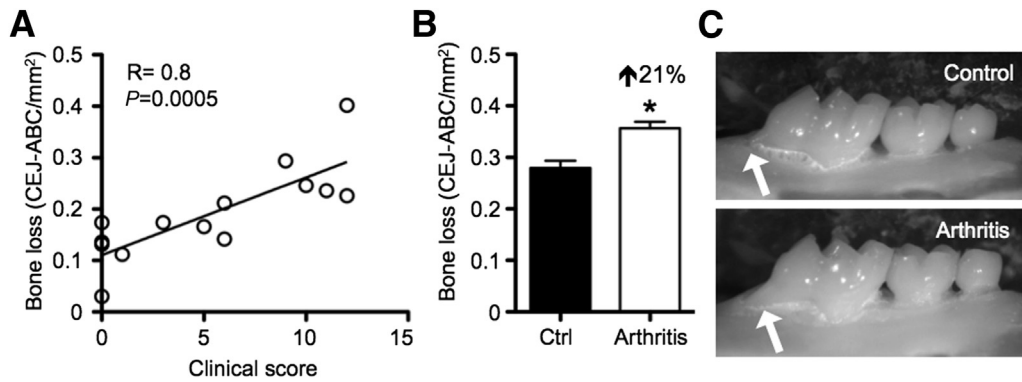
### Aging-Associated Alveolar Bone Loss Is Accelerated in MC<sub>3</sub>-Deficient Mice

The evidence obtained in our previous experiments suggests that activation of MC<sub>3</sub> (the main receptor that mediates DTrp actions<sup>19</sup>) might protect from alveolar bone loss. Because there is evidence in the literature furthering an association between the melanocortin system and bone metabolism,<sup>12</sup> as well as linking normal bone loss associated with aging,<sup>33,34</sup> next we sought to investigate if MC<sub>3</sub> played any role in these phenomena.

Maxillae from WT and *Mc3r*<sup>-/-</sup> mice, harvested at different ages (1.5, 3.5, and 4.5 months old), were analyzed for alveolar bone loss. Interestingly, periodontal bone loss was accelerated in *Mc3r*<sup>-/-</sup> mice, showing a 39% increase at 3.5 months of age compared with younger mice (1.5 months), whereas only a 15% increase was observed in same-age WT mice (Figure 5).

There is some indication for a functional association between MC<sub>3</sub> and osteoclastogenesis, because the number of osteoclasts is increased in *Mc3r*<sup>-/-</sup> arthritic mice, compared

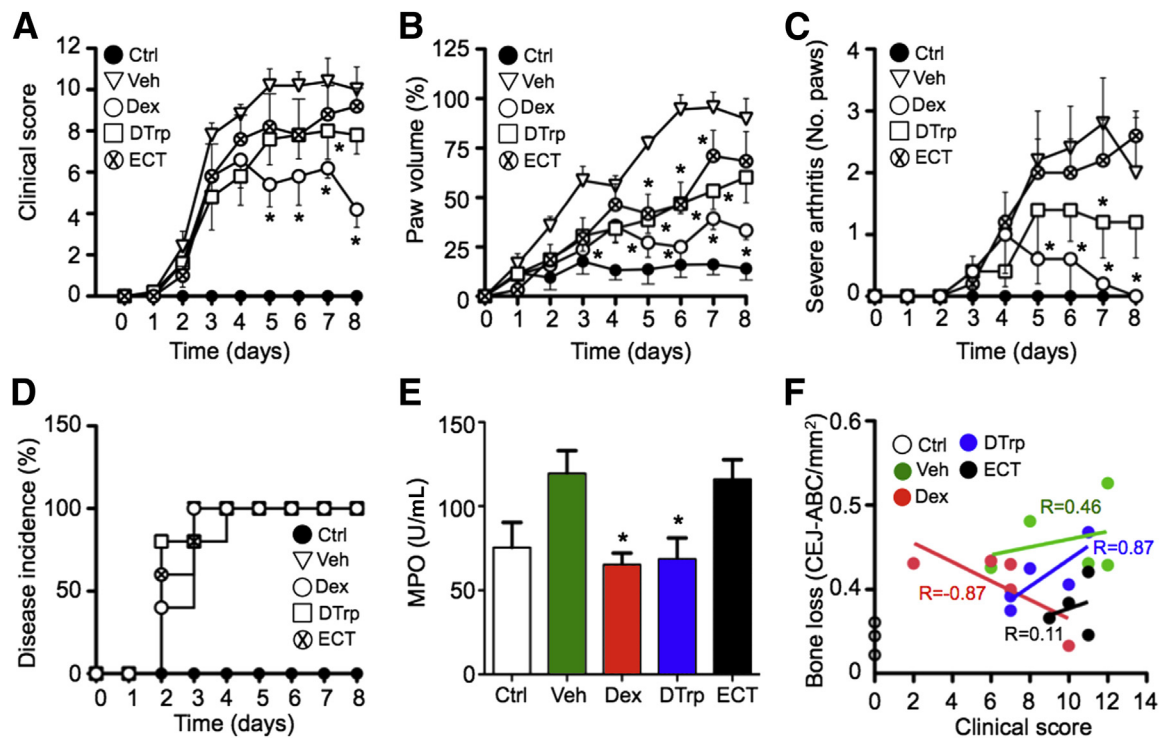




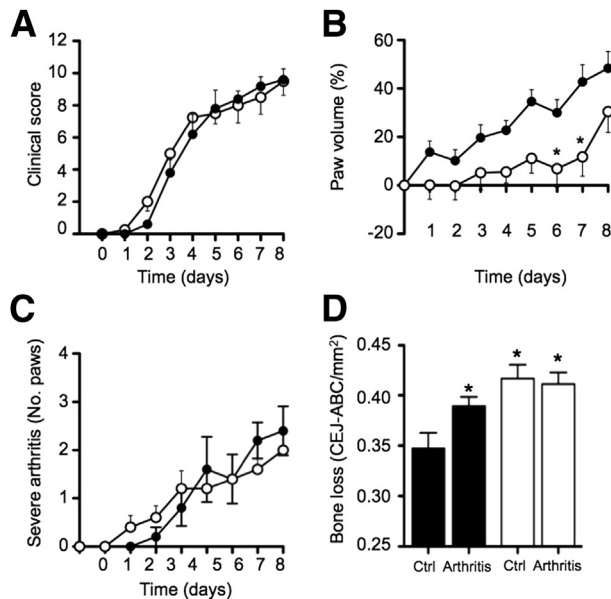
**Figure 2** Correlation of arthritis with alveolar bone loss in the K/BxN serum transfer model. Alveolar bone loss was evaluated at day 7 in the palatal aspect of the first upper molar of the right hemimaxillae. **A:** Correlation between alveolar bone loss and clinical score on mice studied in the protocol comparison experiment (Figure 1) analyzed by Pearson correlation test ( $n = 14$ ). **B:** Overall increase in alveolar bone loss in arthritic mice (pooled data from all mice, means  $\pm$  SEM,  $n = 14$ ) compared with control (Ctrl) mice, analyzed by  $t$ -test.  $*P < 0.05$ . **C:** Representative photographs of the maxillae, showing evidence of alveolar bone loss (arrows).

with WT mice.<sup>18</sup> We then studied if osteoclast numbers were also different in the maxillae of the two genotypes by quantifying the Trap<sup>+</sup> cells in the cervical area of the first molar of the left lower maxillae ( $n = 4$  mice). Although basal levels (1.5 months old) were different, we quantified a 40% increase in Trap<sup>+</sup> cells in older mice in *Mc3r*<sup>-/-</sup> but not in WT mice, where values remained stable (Figure 6A).

We also observed a significant increase in the number of neutrophils in the junctional epithelium of *Mc3r*<sup>-/-</sup> mice compared with WT mice (Figure 6B). However, no differences were found in the rest of epithelial and connective tissue, suggesting that neutrophils might not be playing a crucial role in the alveolar bone loss, as measured in our experimental conditions.



**Figure 3** Effect of dexamethasone, DTrp, and ECT in arthritis and alveolar bone loss. Arthritis was induced using the 100 + 100 protocol (100  $\mu$ L of serum on days 0 and 2) and monitored by daily recording the clinical score (**A**), paw volume (**B**), severity (number of paws reaching the maximum score) (**C**), and disease incidence (**D**). **E:** MPO activity was measured in the left hemimaxillae at day 8. **F:** Alveolar bone loss was analyzed in the right hemimaxillae at day 8 and correlated with clinical score recorded that day. Drugs were administered i.p. once daily: Dex, 10  $\mu$ g per mouse; DTrp, 20  $\mu$ g per mouse; ECT, 30 ng per mouse; and vehicle PBS (Veh). Nonarthritic mice were included as controls (Ctrl). Data are the means  $\pm$  SEM of five to six mice per group. Data were analyzed by two-way analysis of variance, followed by a Bonferroni multiple-comparison test (**A–C**), one-way analysis of variance, followed by a Bonferroni multiple-comparison test (**E**), and Pearson correlation test (**F**).  $*P < 0.05$ .



**Figure 4** Arthritis and alveolar bone loss in MC<sub>3</sub>-deficient mice. Arthritis was induced in C57BL/6J WT mice (black) and melanocortin receptor 3-deficient mice (*Mc3r*<sup>-/-</sup>) (white) using the 100 + 100 protocol (100  $\mu$ L of serum on days 0 and 2). Disease was monitored by daily recording of the clinical score (A), paw volume (B), and disease severity (number of paws reaching the maximum score) (C). Alveolar bone loss was analyzed in the right hemimaxillae on the last day of the experiment (day 8). Data are the means  $\pm$  SEM of five mice per group. Statistical analyses were performed by two-way analysis of variance, followed by a Bonferroni multiple-comparison test (A–C), and one-way analysis of variance, followed by a Newman-Keuls multiple-comparison test versus WT-control (Ctrl; D). \**P* < 0.05.

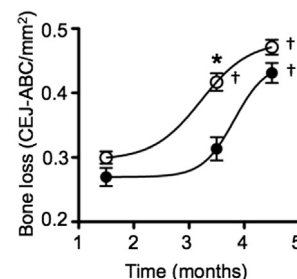
## Discussion

There is clinical evidence that periodontal disease is associated with RA, although it is unclear whether the link is causal or casual. Bone resorption in the maxillae (associated with a high degree of immune cell infiltration) is reminiscent of the RA joint, where many blood-borne cells can be found in the exudate during the active phases of the disease. To gain information on pathogenesis to subsequently inform on therapeutic opportunities, it is important to develop animal models in which disease development could be monitored at the two sites in parallel.

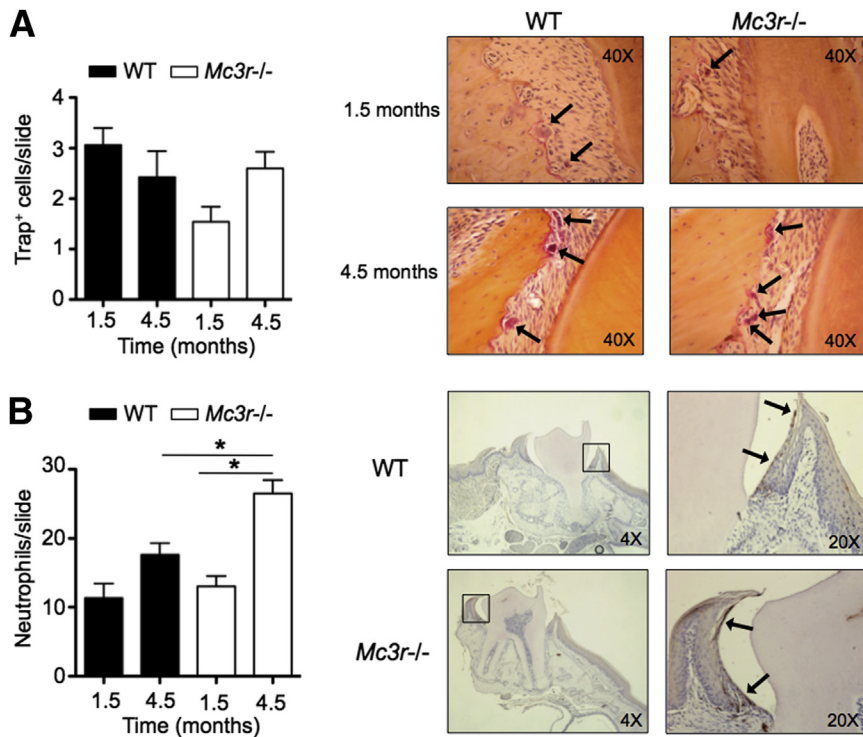
The recent appreciation that mice subjected to the gold standard model of RA, collagen-induced arthritis, develop alveolar bone loss that parallels ankle joint damage,<sup>25</sup> which represents an important conceptual and experimental advance. Park et al<sup>25</sup> reported severe periodontal bone damage 16 weeks after induction of arthritis using type II collagen: this was associated with increased osteoclastic activity and impaired repair ability due to reduced bone formation by osteoblasts. Herein, we used the K/BxN serum model of inflammatory arthritis, an aggressive model that mimics the active phases of RA; also, it is much faster in its onset. Injection of the serum rich in anti-glucose-6-phosphate isomerase immunoglobulins fixes complement onto

cartilage with initiation of an inflammatory reaction, highly reliant on cytokines and eicosanoids.<sup>27,29</sup> Herein, we first determined whether this rapid model (approximately 7 to 8 days versus >30 days for the collagen-induced arthritis) also led to periodontal disease. Thus, administration of the arthritogenic serum along three different protocols induced evident joint inflammation, with more consistent and reproducible data with the 100 + 100  $\mu$ L protocol and mild inflammation when using the 50 + 50  $\mu$ L protocol. A good association was observed between serum dosage and protocol of administration, which resulted in distinct severity of arthritis, and the corresponding degree of alveolar bone loss measured in maxillae. Collectively, these data, coupled to the two recent studies,<sup>24,25</sup> indicate that experimental polyarthritis in rodents is associated with periodontal disease features.

The melanocortin receptor agonist ACTH is an anti-arthritic drug indicated for the treatment of acute inflammatory episodes of gout<sup>35</sup> and RA, as shown by the seminal work of Hench<sup>36</sup> of the Mayo Clinic (Rochester, MN).<sup>37</sup> The equally old observations of Gutman and Yu<sup>6</sup> have been repeated in more rigorous clinical studies, confirming ACTH efficacy in human gouty arthritis.<sup>38–40</sup> All these studies indicate that ACTH is effective and safe for the treatment of acute gout and presents as a good alternative in patients with comorbidities, in whom steroids and colchicine are not recommended. Why is ACTH so effective in arthritides? Ritter et al<sup>40</sup> indicated the possible existence of mechanisms aside from adrenal stimulation and glucocorticoid release, inciting us to identify peripheral modulation of MC<sub>3</sub> as an important contributor of the anti-inflammatory actions of the peptide.<sup>8</sup> However, ACTH is a pan-MC receptor agonist. Herein, we used peptide DTrp, which, although not totally selective in *in vitro* expression cell systems, retains functional selectivity in the mouse, as demonstrated by its lack of efficacy in *Mc3r*<sup>-/-</sup> animals. More important, DTrp displays anti-arthritic effects in the K/BxN animal model of arthritis.<sup>18,19,41</sup>



**Figure 5** Impact of aging on alveolar bone loss in MC<sub>3</sub>-deficient mice. Alveolar bone loss was evaluated in the right hemimaxillae in mice from different ages (1.5, 3.5, and 4.5 months old) in both C57BL/6J WT mice (white circles) and melanocortin receptor 3-deficient mice (*Mc3r*<sup>-/-</sup>) (black circles). Data are the means  $\pm$  SEM of 6 to 17 mice. Data were analyzed by two-way analysis of variance, followed by a Bonferroni multiple-comparison test. \**P* < 0.05 versus 1.5 months; †*P* < 0.05 for WT versus *Mc3r*<sup>-/-</sup>.



**Figure 6** Analysis of osteoclasts and neutrophils in gingival tissues. The left hemimaxillae were used for histological evaluation of osteoclast activity by Trap staining and neutrophil infiltration. **A:** The number of osteoclasts on the cervical area of the first molar. Representative images of Trap<sup>+</sup> cells (arrows). **B:** Sections were stained for neutrophil elastase as a marker of neutrophils. Representative images of 1.5-month-old mice. Arrows designate neutrophils. Data are the means  $\pm$  SEM of two to four mice. Data were analyzed by one-way analysis of variance, followed by a Bonferroni multiple-comparison test. \* $P < 0.05$ .

The pharmacological experiments suggested that MC<sub>3</sub>-based therapy may yield a unique opportunity. Although the glucocorticoid Dex afforded the expected therapeutic effect on the arthritic joint,<sup>18</sup> measured in terms of score, swelling, disease severity, and MPO activity, it did not affect—and rather worsened—alveolar bone loss. This effect can be chiefly due to the osteoclast activating property of glucocorticoids.<sup>42</sup> Calcitonin, on the other hand, was selected because it represented an opposite therapeutic effect, with little modulation of inflammatory arthritis,<sup>43</sup> yet its daily delivery to mice from day 2 significantly protected from alveolar bone loss associated with this model of experimental arthritis. This was predicted in view of the potent action of calcitonin in stopping bone resorption,<sup>44</sup> being able to override the activating effect of glucocorticoids.<sup>43</sup>

DTrp revealed unique properties because it was able to inhibit arthritis, albeit to an intermediate level between Dex and calcitonin, and attenuated bone loss associated with periodontal disease. In more detail, the DTrp group showed a positive high correlation between clinical score and bone loss (ie, reduced bone loss associated with the anti-arthritic effect,  $r = 0.87$ ), and this was the exact opposite of that calculated for Dex-treated mice ( $r = -0.87$ ). This finding is of relevance because prolonged steroid therapy is associated with bone density loss, osteoporosis, and fractures. These results indicate that MC receptor agonists, possibly better if selective for MC<sub>3</sub>, represent a novel class of anti-arthritic therapeutics able to target joint disease without aggravating unwanted effects on distant organs and tissues. This notion is further substantiated by a recent study in which the beneficial effect of melanocortin treatment on joint

inflammation and against systemic muscle wasting (cachexia) was demonstrated.<sup>21</sup>

The bone-protective effect of DTrp is likely due a direct osteoclast effect that is additive to modulation of local inflammation, as shown by the MPO activity measurements in maxillae samples. In agreement with these pharmacological data, we have reported a higher degree of osteoclastogenesis in *Mc3r*<sup>-/-</sup> mice, measured both *in vivo* in arthritic joints and *in vitro*, using bone marrow-derived osteoclasts.<sup>18</sup> Furthermore, in WT osteoclasts, application of DTrp reduced cell activation and resorptive activity. In these experiments, *Mc3r*<sup>-/-</sup> mice did not present more pronounced arthritis, at variance from what we reported previously,<sup>18</sup> likely due to differences in protocol and animal age. By the same token, this new result allows us to separate periodontal bone loss and joint arthritis, indicating that the former occurs independently from modulation of the latter. Collectively, these data prompt us to identify MC<sub>3</sub> as a modulatory receptor on osteoclast differentiation and activation.

The use of genetically engineered mice can shed new insights into the biological functions of genes of interest. The involvement of MC<sub>3</sub> in bone metabolism emerges from pharmacological evidence or from the use of *Mc3r*<sup>-/-</sup> in settings of experimental pathology. But, if MC<sub>3</sub> plays an important nonredundant role in bone physiology, then its absence might produce a phenotype in healthy mice. Indeed, these mice present decreased linear growth and femur length, as well as reduced bone mineral density.<sup>45,46</sup> To this end, we monitored the degree of bone erosion in the maxillae of mice at different ages, comparing WT with *Mc3r*<sup>-/-</sup> animals. These experiments confirmed a higher susceptibility to alveolar bone



loss in the transgenic mice lacking the MC<sub>3</sub> receptor, with presence of significant bone loss as early as 14 weeks of age, whereas WT mice displayed similar degrees of damage at 18 weeks. Thus, endogenous MC<sub>3</sub>, possibly activated by circulating ACTH or  $\alpha$ MSH, exerts a tonic inhibitory role on bone metabolism in the maxillae; hence, in its absence, there is a higher susceptibility to bone loss, hence disease. Although congruent with the data previously presented, this hypothesis requires corroboration by future studies. With aging, *Mc3r*<sup>-/-</sup> mice become obese, an effect evident at 12 weeks of age.<sup>47</sup> Because it is reported that obese mice have a different microbiota compared with lean animals,<sup>48</sup> one could not exclude that a different microbiota predisposes to higher susceptibility to periodontal disease. Again, focused and systematic analyses on the periodontal compartment of MC<sub>3</sub>-deficient mice can shed light onto this novel biology of the MC system we have unveiled herein.

In summary, we describe a novel experimental association between periodontal disease and inflammatory arthritis with two distinct outcomes: first, the modulatory function of MC<sub>3</sub> on periodontal status in health and disease; second, the distinct pharmacology of DTrp compared with other anti-arthritic or bone-protective compounds, suggesting the potential development of a new genus of anti-arthritic therapeutics, centered on MC<sub>3</sub> activation and able to spare or correct alveolar bone damage.

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T.M.-M. designed the study, performed experiments, analyzed and interpreted data, and wrote the manuscript; M.F.M.M. performed experiments, analyzed and interpreted data, and revised the manuscript; L.V.N. and A.A. performed experiments and revised the manuscript; M.A.C. and T.A.d.S. interpreted data and revised the manuscript; and M.P. designed the study, wrote the manuscript, and provided funding.

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